Institut für Veterinärphysiologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. med. vet. Max Gassmann
Stellvertretender Direktor: Prof. Dr. med. vet. Thomas A. Lutz

Arbeit unter wissenschaftlicher Betreuung von:
PD Dr. rer. nat. Thomas Riediger

Lipopolysaccharide inhibits ghrelin-excited neurons and reduces food intake via central nitric oxide signaling

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Sarah Elisabeth Pinkernell

Tierärztin
aus Meppen (Ems), Deutschland

genehmigt auf Antrag von
PD Dr. rer. nat. Thomas Riediger, Referent

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Contents

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"Lipopolysaccharide inhibits ghrelin-excited neurons and reduces food intake via central nitric oxide signaling"

Treatment with lipopolysaccharides (LPS) is a model of inflammatory disease-related anorexia. The neuromodulator nitric oxide (NO) mediates LPS anorexia as demonstrated by peripheral administration of 1400W, which specifically inhibits inducible NO synthase (iNOS). LPS induces iNOS expression in the hypothalamic arcuate nucleus (Arc), where NO inhibits orexigenic (ghrelin-excited) neurons. This study evaluates the hypothesis that central iNOS signaling mediates LPS anorexia. The effect of third intracerebroventricular (icv) injection of 1400W on LPS-induced anorexia was investigated. Furthermore, it was tested immunohistochemically whether LPS triggers the phosphorylation of the transcription factor STAT1, which contributes to iNOS gene expression. Central 1400W infusion reversed LPS anorexia and it attenuated LPS-dependent decreases in energy expenditure and respiratory quotient. Peripheral LPS treatment induced a significant pSTAT1 response in the Arc. In independent electrophysiological studies pharmacological iNOS blockade disinhibited orexigenic Arc neurons after in vivo or in vitro treatment with LPS. In conclusion, central NO signaling seems to contribute to LPS anorexia, possibly by inhibiting ghrelin-excited neurons via iNOS-dependent NO formation. Hence, pharmacological iNOS inhibition might be a therapeutic approach to treat disease related anorexia.

nitric oxide; anorexia; arcuate nucleus; lipopolysaccharide; ghrelin

Vetsuisse-Fakultät Zürich 2012
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“Lipopolysaccharide hemmen Ghrelin-sensitive Neurone und reduzieren die Nahrungsaufnahme durch eine zentrale, Stickstoffmonoxid vermittelte Wirkung”

Die Behandlung mit Lipopolysacchariden (LPS) ist eine anerkannte Methode zur Induzierung krankheitsbegleitender Anorexie. An der Entwicklung der LPS-Anorexie ist Stickstoffmonoxid (NO) beteiligt. LPS bewirkt die Expression der induzierbaren NO-Synthase (iNOS) im hypothalamischen Nucleus arcuatus (Arc), wo NO Ghrelin-aktivierte Neurone hemmt. Wie vorherige Studien zeigen, wirkt eine systemische Verabreichung des spezifischen iNOS-Inhibitors 1400W dem anorektischen LPS-Effekt entgegen. Diese Arbeit untersucht mittels intrazerebroventrikulärer 1400W-Infusion den Beitrag zentral exprimierter iNOS zur Entstehung der LPS-Anorexie. Zudem wurde immunohistochemisch getestet, ob die Phosphorylierung des Transkriptions-Faktors STAT1, welcher zur iNOS-Genexpression beiträgt, durch LPS verstärkt wird. Zentrale 1400W-Applikation schwächt den LPS-Effekt auf Futteraufnahme, Energieumsatz und respiratorischen Quotienten signifikant ab. Elektrophysiologische Untersuchungen bestätigen, dass 1400W die LPS-bedingte Hemmung orexigener Arc-Neurone aufhebt. Periphere LPS-Administration ruft eine gesteigerte STAT1-Phosphorylierung im Arc hervor. Zentrale iNOS-Exprimierung scheint somit zur Entwicklung der LPS-Anorexie beizutragen, wahrscheinlich indem NO orexigene Arc-Neurone hemmt. Daher könnte pharmakologische iNOS-Hemmung eine Therapieoption sein.

Stickstoffmonoxid; Anorexie; Nucleus arcuatus; Lipopolysaccharide; Ghrelin

Lipopolysaccharide inhibits ghrelin-excited neurons of the arcuate nucleus and reduces food intake via central nitric oxide signaling

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Abstract

Lipopolysaccharide (LPS) induces anorexia and expression of inducible nitric oxide synthase (iNOS) in the hypothalamic arcuate nucleus (Arc). Peripheral administration of the iNOS inhibitor 1400W counteracts the anorectic effects of LPS. Here we investigated the role of central NO signaling in LPS anorexia. In electrophysiological studies we tested whether 1400W counteracts the iNOS-dependent inhibition of Arc neurons triggered by in vivo or in vitro stimulation with LPS. We used the hormone ghrelin as a functional reference stimulus that is known to activate orexigenic Arc neurons. Further, we investigated whether in vitro LPS stimulation induces an iNOS-mediated formation of the second messenger cGMP. Since the STAT1 pathway contributes to the regulation of iNOS expression we investigated whether LPS treatment induces STAT1 phosphorylation in the Arc. Finally we tested the effect of intracerebroventricular injection of 1400W on LPS-induced anorexia. Superfusion with 1400W (10^{-4} M) increased neuronal activity in 37% of neurons in Arc slices from LPS treated (100 µg/kg ip) but not from saline treated rats. Similarly, 1400W excited 45% of Arc neurons after in vitro stimulation with LPS (100 ng/ml). In both approaches, a considerable percentage of 1400W sensitive neurons were excited by ghrelin (10^{-8} M; 50% and 75%). In vitro stimulation with LPS induced cGMP formation in the Arc, which was blocked by co-incubation with 1400W. LPS treatment elicited a pSTAT1 response in the Arc of mice. Central 1400W injection (4 µg/rat) attenuated LPS-induced anorexia and counteracted the LPS-dependent decrease in respiratory quotient and energy expenditure. In conclusion, the current findings substantiate a role of central iNOS dependent NO formation in LPS-induced effects on eating and energy homeostasis. A pharmacological blockade of NO formation might be a therapeutic approach to ameliorate disease-related anorexia.

Keywords: nitric oxide; anorexia; hypothalamus; ghrelin; arcuate nucleus; inflammation; lipopolysaccharide; electrophysiology

Introduction

Chronic anorexia is a deleterious consequence of inflammatory diseases, such as bacterial or parasitic infections, cancer, AIDS, kidney and heart failure, rheumatoid arthritis, chronic obstructive pulmonary disease (COPD) and others (Hart, 1988; Plata-Salaman, 1996). Sickness-related anorexia is a condition with negative impact on recovery and treatment success, with increased morbidity and mortality and with a decreased quality of life. The brain represents a major target for pro-inflammatory stimuli like pro-inflammatory cytokines that influence neuronal circuits controlling food intake and energy homeostasis. As part of these inflammatory processes the gaseous neuromodulator nitric oxide (NO) emerged as a possible mediator of sickness anorexia. Treatment with the bacterial endotoxin lipopolysaccharide (LPS), a major constituent of the cell wall of gram-negative bacteria, is a commonly used model for triggering an acute phase response that is associated with pronounced hypophagia (Abram et al., 2000; Langhans, 2007). Previous studies demonstrated an LPS-dependent expression of the inflammatory NO producing enzyme inducible nitric oxide synthase (iNOS) in the hypothalamic arcuate nucleus (Arc) (Wong et al., 1996) which is a key structure implicated in the control of energy homeostasis (Jansky et al., 1995; Woods et al., 1998). The artificial NO donor sodium nitroprusside (SNP) strongly inhibits Arc neurons that are activated by the orexigenic hormone ghrelin (Riediger et al., 2006). Moreover, LPS treatment attenuates fasting-induced activation of Arc neurons suggesting an interaction between hunger signals and LPS (Becskei et al., 2008). These findings lead us to hypothesize that NO might be implicated in the neuroinflammatory processes underlying LPS-induced anorexia.

1400W is a specific and long acting iNOS inhibitor (Garvey et al., 1997). Peripheral 1400W injection attenuates LPS-induced hypophagia and associated disease symptoms such as inactivity, hyperthermia and reduced energy expenditure (Riediger et al., 2010). Although these studies support the concept that NO signaling contributes to LPS anorexia, it remained

to be elucidated whether brain-intrinsic iNOS activity is important for this effect and whether LPS inhibits orexigenic Arc neurons via iNOS-dependent NO formation. In our current study we used electrophysiological, immunohistochemical and behavioral approaches to shed more light on the neuronal effects of NO in the context of sickness anorexia.

Based on the assumption that a blockade of LPS-induced iNOS activity should result in increased neuronal activity of Arc neurons, we conducted in vivo and in vitro stimulations with LPS of rats and brain slices containing the Arc, respectively, and characterized the effects of the iNOS inhibitor 1400W on the electrical activity in the Arc. If our assumption that LPS inhibits orexigenic Arc neurons by induction of iNOS-dependent NO formation were correct, a blockade of endogenous iNOS activity after LPS stimulation should lead to increased neuronal activity, at least in a subpopulation of ghrelin-excited Arc neurons. There is in vitro and in vivo evidence that NPY neurons are directly activated by ghrelin (Dickson and Luckman, 1997; Kohno et al., 2003). Based on these studies it is justified to assume that NPY neurons are the major subpopulation of cells that are excited by ghrelin in the Arc. This is also supported by the fact that 94% Arc neurons expressing the ghrelin receptor are NPY positive (Willesen et al., 1999). In addition to its direct excitatory effect, ghrelin also indirectly inhibits putative pro-opiomelanocortin (POMC) neurons in the lateral Arc (Cowley et al., 2003; Riediger et al., 2003). In the present study, we recorded from the medial Arc, where ghrelin predominantly induces direct excitatory effects on Arc neurons that are directly inhibited by NO (Riediger et al., 2006; Riediger et al., 2003).

As previously reported, prostaglandins might regulate iNOS gene expression (Chen et al., 1999). Furthermore, prostaglandins contribute to the anorectic action of LPS (Langhans et al., 1989; Lugarini et al., 2002). Therefore, we investigated the possible involvement of prostaglandins in the LPS/NO-dependent modulation of neuronal Arc activity. Using indomethacin to block cyclooxygenase (COX)-dependent prostaglandin synthesis we tested

whether prostaglandins are necessary for the presumed LPS-induced NO-dependent inhibition of Arc neurons.

NO modulates neuronal activity and other biological actions by activating the soluble guanylate cyclase (sGC), which catalyzes the conversion of GTP to the second messenger cyclic GMP (Feil and Kleppisch, 2008; Schmidt et al., 1993). In previous immunohistological studies the NO donor sodium nitroprusside (SNP) elicited a cGMP response in the Arc (Riediger et al., 2006). Hence, an LPS/NO-mediated inhibition of Arc neurons is likely to be paralleled by an intracellular cGMP formation. This was tested under experimental in vitro conditions similar to the electrophysiological experiments. 1400W was used to confirm the possible involvement of iNOS in the LPS-mediated cGMP response.

It was a further aim of this study to identify the possible transcriptional processes involved in LPS-induced iNOS expression in the Arc. The JAK-STAT pathway is an important intracellular signaling cascade regulating gene transcription in response to inflammatory stimuli (Lim and Cao, 2006). Among the different STAT isoforms particularly STAT1 seems to contribute to the activation of the iNOS promotor region (Guo et al., 2007). We therefore investigated the effect of peripheral LPS injection on STAT1 phosphorylation in the Arc.

Finally, the ability of 1400W to cross the blood-brain barrier (Garvey et al., 1997) is an important pre-requisite for a potential therapeutic use of this drug and its derivatives. However, this characteristic makes it difficult to dissociate peripheral effects from central modes of action when 1400W is administered peripherally. In order to test whether central iNOS/NO signaling mediates sickness-related anorexia, we investigated the effect of third intracerebroventricular (icv) infusion of 1400W on the LPS-induced suppression of food intake.

Materials and methods

Animals and housing conditions

For the electrophysiological and behavioral studies, male adult Wistar rats (200-300 g) (Elevage Janvier, Le-Genest-St. Isle, France) were used. The animals were housed at controlled temperature (21 ± 1°C) and a 12-h/12-h artificial light cycle with ad libitum access to standard laboratory rat chow (890 25 W16, Provimi Kliba, AG, Kaiseraugst, Switzerland) and tap water. The behavioral and metabolic studies were conducted in an open-circuit indirect calorimetric system (AccuScan Instruments Inc.; Columbus, OH, USA). Rats were single-housed on a layer of wood shavings in Plexiglas cages (42x42x30 cm). Water bottles and food cups were placed on scales to measure water and food intake continuously. Powdered rodent chow (GLP 3433, Provimi Kliba, AG, Kaiseraugst, Switzerland) was used to avoid food hoarding and in order to precisely detect small amounts of eaten food.

Male C57BL/6 mice bred in our local animal facility were used for the immunohistological detection of LPS-induced STAT1 signaling. Mice were group-housed in standard macrolon cages on a layer of wood shavings under a 12-h/12-h light-dark cycle with free access to rodent chow and water. All animals were handled and adapted to the experimental conditions before the onset of the experiments. The Veterinary Office of the Canton Zurich approved all experiments.

Electrophysiology studies

The electrophysiological recording technique was described previously (Riediger et al., 2004; Riediger et al., 2006; Riediger et al., 2003; Traebert et al., 2002). Briefly, the rats were decapitated using a guillotine and their brains were quickly removed and superfused with ice-cold artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl 124; KCl 5; NaH_2PO_4 1,2; $MgSO_4$ 1,3; $CaCl_2$ 1,2; $NaHCO_3$ 26; glucose 10; oxygenated and pH-equilibrated with oxycarbon (95% O_2, 5% CO_2); pH 7,4; 290 mosm/kg. 700 µm thick coronal brain slices were cut at the mid-rostral level of the Arc using a vibratom (Leica VT1000S, Leica Microsystems, Germany). A rectangular 3x3 mm slice preparation containing the Arc was manually dissected under a dissection microscope and transferred to a temperature controlled (37°C) incubation chamber filled with constantly oxygenated aCSF. For recordings, the Arc preparations were then transferred to a temperature-controlled (37°C) recording chamber that was constantly perfused with oxygenated and pre-warmed aCSF at a rate of 1,6 ml/min. Extracellular single-unit recordings were obtained using self-made glass-coated platinum-iridium electrodes. According to the neuroanatomical brain map (Paxinos and Watson, 2007), recordings were conducted in the medial arcuate nucleus (ArcM), in which the majority of neurons are excited by ghrelin (Riediger et al., 2003).

In order to test whether LPS modulates the neuronal Arc activity via iNOS-dependent induction of NO, two different approaches were used. For the in vivo stimulation with LPS, rats received an intraperitoneal (ip) injection of 100 µg/kg LPS (from Escherichia coli, Sigma Aldrich, Switzerland) during the second half of the light phase and 4 hours before the animals were decapitated as described above. For the in vitro stimulation, brains from untreated rats were collected and prepared as described above. The slices were then incubated for 4 h in 240 ml of aCSF in an incubation chamber containing 100 ng/ml of LPS. The sensitivity to the iNOS inhibitor 1400W (10^{-4} M; Calbiochem, Switzerland), the NO donor SNP (10^{-5} - 10^{-4} M;

Calbiochem, Switzerland) and rat ghrelin (10^{-8} M; Bachem, Switzerland) was tested by switching to an aCSF solution containing these substances. The aCSF used for the recordings did not contain LPS.

In order to test whether the LPS/NO-induced effect on Arc activity depends on prostaglandin signaling, the COX inhibitor indomethacin (10^{-5} M) was co-incubated in vitro with LPS for 4 h prior to electrophysiological recordings. At the end of the incubation period, the Arc preparations were transferred to the recording chamber and the sensitivity of Arc neurons to 1400W, SNP and ghrelin was tested as described above.

Immunohistological studies

LPS induced iNOS-dependent cGMP formation in the Arc

The effect of LPS on the intracellular formation of cGMP was investigated in Arc slices that were prepared under the same conditions as those in the electrophysiological studies. Arc sections were incubated for 4 h in aCSF containing LPS (100 ng/ml), LPS + 1400W (10^{-4} M), 1400W or vehicle. In order to prevent the degradation of cGMP, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (10^{-5} M, IBMX, Sigma-Aldrich, Switzerland) was added after 2.5 h to the incubation medium. At the end of the total 4 h incubation period, Arc preparations were transferred to a 12-well plate filled with 2 ml of oxygenated aCSF. Incubations were terminated by adding 2 ml of ice-cold paraformaldehyde (PFA) solution (final concentration of 4% PFA in PBS, 1 h, 4°C). The fixative was replaced with ice-cold PFA solution containing 10% sucrose in phosphate buffered saline (PBS; 4°C, 2 h), followed by 10% sucrose in PBS (4°C, 30 min). 20 µm-thick frozen coronal sections were cut in a cryomicrotome (CM3050S, Leica Microsystems, Germany), thaw-mounted on microscopic glass slides (Superfrost Plus, Faust, Switzerland) and stored at -20°C. Sections were air-dried for 1 h at room temperature and subsequently rehydrated (3 x 5 min) with PBS containing

0.1% Triton® X-100 (PBST). The sections were then incubated with the primary antibody (anti-sheep cGMP antibody, 1:6000 in 0.3% PBST, kindly provided by Jan DeVente, University of Maastricht, Netherlands) for 24 h at 4°C. After washing with 0.1% PBST (3 x 10 min) the sections were incubated with the secondary antibody at room temperature for 75 min (Alexafluor 488 donkey-anti-sheep, Invitrogen, USA, 1:200 in 0.3% PBST). After final washing in 0.1% PBST (3 x 10 min) the slides were coverslipped with PBS/glycerol (1:1 v/v; Citifluor, Citifluor Ltd, UK).

LPS induced STAT1 phosphorylation in the Arc

Mice weighing approximately 35 g were subcutaneously (sc) injected with either LPS (40 μg/mouse, dissolved in saline) or saline (100 μl/10g BW) at dark onset. The LPS dose was adopted from our previous immunohistochemical and behavioral studies (Becskei et al., 2008). 4 hours after injection the mice were anesthetized by an injection of pentobarbital (pentobarbital sodium, 80 mg/kg, ip). The thorax was opened and the mice were transcardially perfused for 2 min with 0.9% NaCl followed by ice-cold PFA solution (2% in 0.1 M PB). Brains were removed and postfixed in ice-cold 2% PFA for 1 h and cryoprotected in 0.02 M potassium phosphate buffered saline (KPBS) containing 20% sucrose for the following 48 h at 4°C. Subsequently brains were snap-frozen at -20°C in hexane. Arc sections (20 μm) were cut in a cryomicrotome, thaw-mounted on microscopic glass slides and stored at -20°C until further processing.

To detect pSTAT1 immunoreactivity the sections were air dried for 1 h and rehydrated in 0.02 M KPBS. Antigen retrieval was achieved by 20 min incubation in 0.02 M KPBS 0.3% NaOH and 0.3% H_2O_2. Afterwards sections were incubated in 0.3% glycine (10 min) followed by 0.03% sodium dodecyl sulfate (SDS, 10 min, both in 0.02 M KPBS). Unspecific binding was blocked by 20 min incubation in blocking solution (KPBS containing 4% donkey normal

serum (DNS), 0.4% Triton® X-100, 1% bovine serum albumin (BSA)). The primary antibody (rabbit anti-pSTAT1 (Tyr 701), 1:50, Santa Cruz Biotechnology) was applied for 48 h at 4°C in KPBS containing 1% DNS, 0.4% Triton® X-100 and 1% BSA. The secondary antibody (donkey anti-Rabbit Alexa 555 1:100, Invitrogen; diluted in KPBS containing 1% DNS and 0.3% Triton® X-100) was applied for 2 h at room temperature. After final washing in 0.02 M KPBS the sections were coverslipped with citifluor.

We also studied the effect of LPS on STAT1 phosphorylation in rats, but for unknown reasons we did not obtain a satisfactory immunohistochemical pSTAT1 signal at least under the current experimental conditions (not shown). Therefore, only the results obtained in mice are reported.

Behavioral studies

Effect of central iNOS inhibition on LPS-induced anorexia and metabolic parameters

Rats weighing approximately 300 g were anesthetized with ketamin/xylazin (0,1 ml/100 g ip, 0.66 mg xylazine and 6.66 mg ketamine/100 g BW) and placed in a stereotaxic device. Throughout surgery the rats were kept under isoflurane anesthesia.

A 22-gauge guide cannula (C313G/Spc cut 9 mm, Plastics One, Roanoke, VA) was implanted into the third ventricle and anchored by dental cement and stainless screws. The coordinates for cannula placement were obtained from the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007): anterioposterior: 2.2 mm posterior to bregma, lateral: ± 0.0 mm, dorsoventral: -7.5 mm). The animals were allowed 5-7 days of recovery after surgeries before the proper position of the guide cannula was confirmed by performing an angiotensin II stimulation test. Angiotensin II (10 ng/2 µl) was injected into the third ventricle with a 10 µl Hamilton syringe connected via polyethylene tubing to the injector that was inserted into the guide cannula. Cannula placement was considered successful if the animal consumed at least

5 ml of water during one hour after injection. Animals that did not fulfill this criterion were excluded from the study.

Rats that passed the angiotensin II test were allocated to two groups. In each of these groups two treatments were conducted in a crossover design with a six-day interval between the trails. In the first group the rats received saline icv/saline ip or saline icv/LPS ip. In the second group, the rats were treated with 1400W icv/saline ip or 1400W icv/LPS ip. This paradigm was chosen to avoid repeated LPS treatments resulting in the development of LPS tolerance (Langhans et al., 1993; Riediger et al., 2010). LPS (100 μg/kg, ip) and 1400W (4 μg/rat in 2 μl) were dissolved in sterile saline; treatments were conducted within 20 min before dark onset.

Over 12 h, dark phase food intake and respiratory gas exchange was measured as described previously (Riediger et al., 2010) in order to calculate total energy expenditure (EE) and respiratory quotient (RQ). Food consumption was measured in 5 minutes intervals and saved on a computer. For EE and RQ measuring, ambient air was pumped through the cages via a manually adjustable flow controller (flow rate set to 2 l/min). Air entering and leaving each cage was monitored for its O_2 and CO_2 concentration. The cages were connected to two analyzers measuring O_2/CO_2 concentration of each cage for a period of 30 s in five minutes intervals, which allowed calculating EE and RQ using the manufacturer's software (PhysioPlot Version 1.80, Integra ME Version 2.21; AccuScan Instruments Inc.). This calculation was based on the following equation: total energy expenditure (kcal/kg/h) = (3.9 x V (O_2) + 1.1 x V (CO_2))/1000; V (O_2) and V (CO_2); data were normalized for body weight.

Statistical analysis

From the constantly recorded rate meter counts, the mean spontaneous baseline activity of each single neuron was evaluated for 60 s before the stimulus (spontaneous activity). This

value was used to normalize changes in the firing rate expressed as absolute mean response change (Hz) and as percentage of mean response change. If both the absolute and the percentage of discharge rate changes during the response were larger than ±0.5 Hz and ±20%, respectively, the neuron was considered sensitive to the applied substance. Furthermore, the latency, the duration and the peak response (Hz) were also measured. The effect parameters of the electrophysiological responses were averaged and expressed as means ± standard errors (M±SE). Comparisons between different proportions of 1400W sensitive neurons observed under the different treatment conditions were made using the Fisher's exact test. Mean spontaneous baseline activity of 1400W sensitive and insensitive neurons was compared using the Mann-Whitney-Wilcoxon test.

For the quantification of the number of cGMP and pSTAT1 immunoreactive cells, a fluorescent microscope equipped with a digital camera system (AxioImager, Carl Zeiss AG) was used. The number of cGMP labeled cells of 5 corresponding Arc sections and the number of pSTAT1 labeled cells of 2-3 corresponding Arc sections from each animal were analyzed manually in blind fashion. From these values the mean number of positive cells per section was calculated for each animal. Statistical comparisons between treatment groups were performed using the one-way analysis of variance (ANOVA) for multiple groups (cGMP) and unpaired t-test for comparison between two groups (pSTAT1).

For statistical evaluation of the behavioral and metabolic parameters the differences between the LPS treated groups relative to their respective control groups were calculated at each time point. These values were compared using parametric unpaired t-test. All data are presented as mean ± SEM, $p < 0.05$ was considered significant.

Results

Electrophysiological studies

In each of the electrophysiological approaches summarized below (in vitro or in vivo stimulation with LPS; in vitro studies involving LPS/indomethacin) separate recordings, Arc preparations and controls have been used. Therefore all of the pertinent data sets are independent. The mean amplitude of the action potentials for all recorded neurons was 120 μV, which allowed an accurate discrimination from background noises.

In line with the assumption that LPS induces iNOS-dependent NO signaling the Arc, the iNOS inhibitor 1400W induced excitatory effects in ghrelin-excited Arc neurons from LPS pre-treated rats (Fig. 1A). The responses started with a mean latency of 428 ± 117 s and seemed to be either irreversible or slowly reversible. Neurons that were excited by 1400W after LPS stimulation also often tended to show reduced spontaneous baseline activity compared to neurons from saline treated rats (Fig. 1A and Fig. 1B). During the stimulatory responses the mean absolute increase in firing was 1.5 ± 0.6 Hz from a mean spontaneous baseline activity of 0.7 ± 0.5 Hz (Table 1). In contrast to the excitatory effects of 1400W after LPS treatment, 1400W did not affect neuronal activity in Arc neurons from saline pre-treated control animals (Fig. 1B), except in one single neuron showing an excitatory response. To confirm NO sensitivity under control conditions, the NO donor SNP was superfused, which caused pronounced inhibitory responses in 86% of the tested cells (Fig. 1B). Neurons from LPS treated rats that increased their firing rate after 1400W displayed a significantly lower spontaneous baseline firing rate than neurons from LPS treated rats that were insensitive to 1400W (Fig. 2). This indicates that the former neurons were tonically inhibited.

In total, 37% (7/19) of all neurons tested from LPS treated rats were excited in response to 1400W (Fig. 3A). One of the activated neurons showed a biphasic response with an excitatory

component at the beginning of the response followed by an inhibitory effect. In Arc neurons from control animals only 4% (1/23) of cells were activated by 1400W. The proportion of 1400W sensitive neurons was significantly higher after LPS treatment than under control conditions.

LPS not only induced inhibitory NO signaling in the Arc after in vivo stimulation but also under in vitro conditions. The outcome of the electrophysiological experiments conducted after in vitro incubation of Arc slices with LPS was similar to the in vivo observations described above. As shown by the representative recordings in Figure 4A, iNOS inhibition by 1400W induced a marked increase in neuronal firing of Arc preparations that were incubated in LPS (for effect parameters see Table 1). This was not the case under control conditions of Arc slices pre-incubated with saline; this lack of responsiveness was not due to NO insensitivity as confirmed by superfusion of the NO donor SNP (Fig. 4B).

While under control conditions only one out of 11 neurons (9%) increased its firing rate in response to 1400W, 45% (4/9) of the tested cells showed an increased activity after 1400W administration and one neuron (11%) was inhibited (Fig. 3B). This difference in the proportions of activated cells was significantly different. Similar to the experiments conducted after in vivo treatment with LPS, the majority of neurons showed inhibitory responses to SNP (66%) while one neuron was excited. Interestingly, the SNP-excited neuron was inhibited by 1400W. Although this type of response characteristic was exceptional, the opposing effects of iNOS blockade and exogenous NO administration were consistent.

Similar to our previous electrophysiological studies from medial Arc neurons, ghrelin predominantly induced excitatory responses. 1400W/ghrelin co-sensitivity was tested in 16 neurons after in vivo LPS treatment and in 9 cells after in vitro LPS stimulation. Excitatory ghrelin responses occurred in 44% and 56% of these recordings. Among the 1400W-excited

neurons 50% and 75%, respectively, were excited by ghrelin. Most of the remaining 1400W-excited cells were insensitive to ghrelin because ghrelin-induced inhibitions are not frequently observed in the medial Arc of rats (around 11% of all recordings in the current experiments).

In order to test whether prostaglandin signaling is required for the LPS/iNOS-dependent inhibition of Arc neurons, Arc slices were incubated in vitro with LPS in combination with or without the COX inhibitor indomethacin, which blocks prostaglandin synthesis.

As shown by the representative recordings in Figure 5, 1400W induced excitatory responses after LPS incubation in the presence or absence of indomethacin indicating that prostaglandin signaling does not seem to be required for the LPS-dependent iNOS induction. Although the percentage of 1400W responsive neurons was slightly lower after COX blockade (33%; 6/18 cells) the proportion of 1400W responsive cells was not significantly different when compared to the positive control conditions without indomethacin (45%; 5/11 cells) (Fig. 3C). The effect parameters for the responses induced by 1400W and the co-sensitivity to ghrelin were similar to the experiments involving LPS in vitro incubation described above (data not shown).

Immunohistological studies

LPS induced iNOS-dependent cGMP formation in the Arc

To investigate whether LPS elicits NO-dependent cGMP formation in the Arc, slice preparations were incubated under comparable in vitro conditions as in the electrophysiological studies. As shown in Figure 6, LPS induced an increase in the number of cGMP immunoreactive cells in the Arc (Fig. 6A), while under control conditions low cGMP immunoreactivity was observed (Fig. 6B). Co-incubation with 1400W abolished the LPS-dependent cGMP formation indicating mediation by iNOS (Fig. 6C). 1400W did not affect

the number of cGMP positive cells in the Arc when applied without LPS (Fig 6D). A significant LPS-induced cGMP response was only observed in the positive control conditions, but not in the presence of 1400W (Fig. 6E).

LPS induced STAT1 phosphorylation in the Arc

Peripheral injection of LPS triggered a pronounced STAT1 phosphorylation in the Arc of mice. Figure 7 shows representative immunostainings of Arc sections. The number of pSTAT1 immunoreactive cells was low in the saline treated control group. LPS treated mice showed a significantly increased number of pSTAT1 positive cells.

Behavioral studies

Effect of central iNOS inhibition on LPS-induced anorexia and metabolic parameters

Compared to control conditions, LPS induced a strong anorectic response during the dark phase. In rats that received central 1400W infusion the LPS-mediated suppression of food intake was completely reversed during the first half of the dark phase and highly markedly attenuated during the rest of the activity phase (Fig. 8A). 1400W alone did not alter on food intake compared to control animals not receiving the iNOS inhibitor. The anorectic response to LPS was significantly attenuated in 1400W-treated animals for most time points during the dark phase when compared to the LPS-mediated feeding inhibition in rats without central iNOS blockade (Fig. 8B).

Similar to the reversal of LPS-induced anorexia, 1400W also partly attenuated the gradual decrease in energy expenditure caused by LPS treatment. Although the LPS-dependent decrease in energy expenditure was clearly reduced in 1400W treated rats at all time points, statistical significance was only reached at 2 h after dark onset (Fig. 8C and 8D). Central

administration of 1400W alone did not seem to affect energy expenditure under the current experimental conditions.

The LPS-mediated reduction in energy intake and expenditure was associated with a clear decline in the RQ reflecting increased fat metabolism (Fig. 8E and 8F). The RQ of 1400W/saline treated rats closely paralleled the values of saline/saline treated controls while the LPS-induced decline in the RQ was markedly attenuated in LPS treated rats with pharmacologically inhibited central iNOS signaling; the 1400W-mediated blockade of the LPS-induced shift towards fat utilization was significant at most time points during the second half of the dark phase. During the first 6 h, the 1400W-induced attenuation of LPS on RQ did not reach statistical significance. The fact that the absolute RQ value in LPS treated rats dropped below the physiological minimum value of 0.7 was probably related to a slight mismatch in the empirical calibration approach defining the RQ of a 24 h fasted rat as 0.7. However, this did not affect the measured change in RQ induced by LPS treatment relative to control conditions.

Discussion

Electrophysiological studies

The main aim of the electrophysiological studies was to show that LPS reduces the activity of Arc neurons by stimulating the iNOS-dependent NO production. The current study confirms this hypothesis and provides electrophysiological evidence that LPS stimulates endogenous iNOS dependent NO signaling in the Arc leading to a decreased activity of Arc neurons. This conclusion is based on the observation that the specific iNOS-inhibitor 1400W increased neuronal activity in the Arc after LPS-stimulation, while hardly any 1400W effect was seen under control conditions. After both, in vivo and in vitro LPS stimulation, a significant percentage of Arc neurons (37% and 45% respectively) were excited by 1400W indicating that LPS inhibits Arc neurons via induction of iNOS expression. Importantly, a there was a considerable co-sensitivity of 1400W-excited neurons to ghrelin (50% and 75%), which was similar to the previously reported co-sensitivity between leptin and ghrelin under comparable experimental conditions (Traebert et al., 2002).

In previous immunohistochemical and electrophysiological studies the inhibitory effects of LPS and the nitric oxide donor SNP on Arc activity were demonstrated (Becskei et al., 2008; Riediger et al., 2010; Riediger et al., 2006). However, it had not been studied so far whether LPS inhibits Arc neurons via a stimulation of endogenous NO. Therefore, the current study extends the previous findings and supports the idea that NO might represent an endogenous anorectic signal in the Arc under inflammatory conditions.

The majority of 1400W-mediated effects were irreversible or slowly reversible during the time of recording, which is consistent with the long-lasting effect of 1400W inhibition on enzymatic iNOS activity (Garvey et al., 1997). In addition to the indirect evidence that LPS

inhibits ghrelin-excited Arc neurons via iNOS-dependent NO release (disinhibitory effect of 1400W), our findings also directly support this mechanism. Arc neurons from LPS treated rats that increased their activity in response to iNOS blockade showed a reduced baseline activity compared to 1400W insensitive neurons and also compared to the baseline activity of neurons from control rats that did not receive LPS. The fact that LPS treatment selectively toned down the activity of neurons that are 1400W responsive under these conditions strongly supports the hypothesis that LPS inhibits Arc neurons via NO formation.

The observation that 1400W was almost ineffective under control conditions without LPS stimulation is consistent with low baseline iNOS activity in the Arc under unstimulated conditions. iNOS expression can be triggered by inflammatory stimuli (Kobayashi, 2010) and is supposed to be virtually absent in the brain under non pathological conditions (Wong et al., 1996). A small percentage of the cells tested under control conditions were also sensitive to 1400W. There are different possible explanations for this finding. On one hand there might be some basal expression of iNOS in the Arc that has not been detected with other methods. On the other hand it also cannot be excluded that some iNOS expression is triggered by the preparation of the Arc or during the subsequent storage of the brain slices in the incubation chambers. Irrespective of the underlying causes, this observation does not confound the main conclusion that LPS induces a NO-dependent inhibition of neuronal Arc activity as reflected by the significantly higher number of 1400W-excited neurons after LPS stimulation relative to control conditions.

In the current study 50% and 75% of neurons responding to 1400W after in vivo and in vitro LPS stimulation, respectively, were also excited by ghrelin. This is an important finding, which might suggest that orexigenic (ghrelin-excited) Arc neurons belong to the target cells for LPS-induced NO signaling. Based on immunohistological and electrophysiological

evidence (Cowley et al., 2003; Traebert et al., 2002; Willesen et al., 1999) the vast majority of ghrelin-excited Arc neurons express NPY. Therefore, it is tempting to speculate that the inhibitory effect of NO on these neurons might decrease the release of the orexigenic neuropeptide NPY in other hypothalamic nuclei (e.g. lateral hypothalamic area (LHA), PVN). Studies showing a restoration of food intake after icv NPY administration in LPS treated animals are in line with this hypothesis (Edwards et al., 1999).

Surprisingly, a low number of ghrelin-inhibited neurons (2/28) showed an increase in their activity in response to iNOS blockade after in vitro LPS stimulation. The functional relevance of this effect is unclear at present. The effect of NO on ghrelin-inhibited Arc neurons has not yet been specifically investigated. Although there is evidence that LPS seems to activate POMC/CART in the Arc (Sergeyev et al., 2001) the role of LPS-dependent nitric oxide signaling in these cells remains to be elucidated.

In our studies we also observed some 1400W-responsive neurons that were insensitive to ghrelin. It cannot be excluded that these cells are also involved in the control of food intake. Based on the neurochemical and functional diversity of Arc neurons, it is reasonable to assume that inflammatory NO signaling might also affect other Arc-dependent effector pathways. The possible function of these cells remains to be identified in future studies.

The electrophysiological results obtained after in vivo and in vitro stimulation with LPS are consistent. However, it is important to consider that in these two approaches, different inflammatory mechanisms might have contributed to the iNOS-mediated inhibition of Arc neurons. While after in vivo LPS injection a peripheral cytokine response is triggered (Jansky et al., 1995; Langhans, 2000), such an effect cannot occur when the Arc is isolated and stimulated under in vitro conditions. This does not necessarily exclude that the LPS/NO response in the Arc is independent of cytokine signaling because LPS may also induce a local cytokine production in the brain parenchyma or in structures of the blood-brain barrier (e.g.

endothelial cells) (Konsman et al., 2004). It was beyond the scope of this study to dissociate direct effects of LPS on NO signaling in the Arc from indirect cytokine-mediated effects. Nevertheless, it can be concluded that a local action of LPS either in structures of the BBB or within the brain tissue itself is sufficient to elicit a NO dependent modulation of neuronal Arc activity.

Similar to cytokines, prostaglandins play an important role in the mediation of LPS anorexia (Lugarini et al., 2002; Pecchi et al., 2009). Therefore we investigated whether a blockade of the prostaglandin-producing enzyme COX interferes with the LPS/NO-mediated modulation of neuronal Arc activity. COX blockade did not prevent the excitatory effect of 1400W on Arc neurons after LPS incubation. In fact the number of 1400W sensitive cells was similar with or without COX inhibition. This suggests that prostaglandins are not required for the LPS-dependent induction of NO signaling in the Arc under our test conditions but these results do not rule out that COX/prostaglandin signaling modulates Arc activity independently of NO. Although the current experiments were not designed to test this, a similar approach could be used to investigate whether superfusion of indomethacin leads to a change in neuronal activity in the Arc after in vivo or in vitro stimulation with LPS.

Immunohistological studies

LPS induces iNOS-dependent cGMP formation in the Arc

We hypothesized that the LPS-dependent induction of NO in the Arc is mediated via cGMP signaling. The current results support this assumption because LPS led to an increase in the number of cGMP positive cells in the Arc. This effect was mediated by iNOS because 1400W completely blocked the LPS-mediated cGMP formation. Notably, the cGMP response in the Arc was comparable to previous studies demonstrating a cGMP formation in the Arc after

incubation with the NO donor SNP (Riediger et al., 2006). Similar to these studies cGMP positive neurons were clearly present in the medial subregion of the Arc corresponding to the localization of the recording site in the electrophysiological experiments. Previous electrophysiological studies demonstrated that the membrane-permeating analog 8-Br-cGMP mimics the inhibitory effect of NO on ghrelin-excited Arc neurons (Riediger et al., 2006). Moreover, the inhibitory action of the NO donor SNP was blocked by the sGC-inhibitor ODQ. Together these findings strongly suggest that the intracellular second messenger cGMP mediates the LPS/NO-dependent Arc inhibition.

LPS induces STAT1 phosphorylation in the Arc

We and others previously demonstrated a time-dependent STAT3 phosphorylation in the Arc after LPS injection (Gautron et al., 2002; Riediger et al., 2010; Rummel et al., 2005). Notably, this effect did not occur in rats that were made LPS tolerant by repeated LPS treatments (Langhans et al., 1993). Hence, the loss of LPS anorexia appears to be associated with the absence of LPS-induced STAT3 phosphorylation (Riediger et al., 2010). Our current study is the first to demonstrate that LPS also triggers a pSTAT1 response in the Arc. For experimental reasons we only report an LPS-induced STAT1 phosphorylation in mice. Based on the similarity of the general neurophysiology of the Arc in rats and mice there is no indication that the inflammatory neuromechanisms that were investigated differ fundamentally between these species. We did not attempt to block the pSTAT1 response with 1400W because pSTAT1 is a known regulator of iNOS gene expression and therefore proximal to iNOS in the LPS-STAT-iNOS signaling cascade. Hence, at least within the framework of our hypotheses, we would not expect any effect of 1400W on the LPS-induced STAT1 phosphorylation. There is cumulating evidence that iNOS expression is linked to STAT1 signaling. The iNOS promoter region is flanked by cytokine responsive DNA motifs.

One of these binds STAT1 and upregulates iNOS gene transcription (Guo et al., 2007). A STAT1-mediated induction of iNOS expression by cytokines and LPS has been demonstrated in different cell systems including astroglial and epithelial cells (Dell'Albani et al., 2001; Kleinert et al., 1998; Stempelj et al., 2007; Tedeschi et al., 2003). It therefore appears likely that LPS-induced STAT1 phosphorylation occurs in these cell types in the Arc similar to what has been demonstrated for STAT3 (Kim et al., 2002; Rummel et al., 2006). It might be interesting to determine the phenotype of the pSTAT1 positive cells. It has to be noted, however, that NO acts as a diffusible neuromodulator in a paracrine manner. Therefore, it can reach and act on neurons within the Arc irrespective of the cell types that release NO in response to LPS.

In contrast to a comparably high basal abundance of pSTAT3 immunoreactive cells in the Arc of rats and mice (Becskei et al., 2010; Riediger et al., 2010), the number of pSTAT1 positive cells was much lower under control conditions. Hence, physiological non-inflammatory stimuli inducing basal STAT3 phosphorylation do not seem to evoke pSTAT1 under the same experimental conditions. Whether pSTAT1 signaling in the Arc is more specific to inflammatory stimuli than pSTAT3 has not yet been elucidated. Similarly, it is possible that the profiles of targets genes regulated by pSTAT3 and pSTAT1 are different. While genetic disruption of STAT signaling bears experimental problems (lethality, altered immune status, etc.) (Takeda and Akira, 2000) a pharmacological blockade of STAT signaling might be an experimental approach to confirm the involvement of pSTAT signaling in iNOS gene expression in the Arc and in LPS anorexia.

Behavioral studies

Effect of central iNOS inhibition on LPS-induced anorexia and metabolic parameters

Proceeding from our previous studies showing an attenuation of LPS anorexia after peripheral 1400W treatment (Riediger et al., 2010) we now provide evidence that central iNOS/NO signaling is involved in sickness anorexia. Central infusion of 1400W blocked the suppressive effect of LPS on food intake and attenuated the LPS-induced decreases in RQ and energy expenditure. Under our test conditions and using our doses (4 μg/rat), the anti-anorectic effect of 1400W appeared to be even stronger than after peripheral administration, because LPS anorexia was completely blocked at least during the first half of the activity phase. It is conceivable that the attenuation of LPS anorexia after peripheral 1400W administration is probably due to a central effect because 1400W crosses the blood-brain barrier (Garvey et al., 1997).

Our current electrophysiological and immunohistological studies point to a possible involvement of the Arc in disease-related anorexia. However, other hypothalamic structures might also be involved. The paraventricular nucleus (PVN) shows a high iNOS expression during inflammation (Wong et al., 1996) and is therefore also a potential brain area where inflammatory NO signaling might affect food intake. Future studies using site-specific injections of 1400W could help to confirm the importance of single brain areas in NO-dependent anorexia.

To our knowledge there are no published studies investigating the effect of centrally administered specific iNOS-inhibitors on LPS-induced anorexia. In several previous studies the effect of unspecific NOS-inhibitors on food intake was tested under non-pathological conditions. Central applications of the unspecific NOS-inhibitors NG-nitro-L-arginine methyl ester (L-NAME) or NG-nitro-L-arginine lead to a decrease in food intake (De Luca et al., 1995; Squadrito et al., 1993). These findings are not necessarily contradictory to our results

because NO has a well-established function in the control of food intake under non-inflammatory physiological conditions (Morley et al., 2011). Various studies have shown that the actions of orexigenic neuropeptides and hormones including orexin-A, NPY and ghrelin depend at least in part on nitric oxide (Farr et al., 2005; Gaskin et al., 2003; Morley et al., 1999). Even if the exact mechanism and the involved brain structures mediating these NO actions remain to be identified, these effects are thought to be primarily mediated by the constitutive NOS isoforms. iNOS is supposed to be absent in the brain under these non-inflammatory conditions (Wong et al., 1996). Therefore, a non-specific NOS inhibition that includes a blockade of nNOS and eNOS may lead to a decrease in food intake under non-pathological conditions (De Luca et al., 1995; Squadrito et al., 1993). The fact that we did not observe an effect caused by 1400W alone but only reversal of LPS anorexia supports the specificity of this iNOS inhibitor.

Similar to our recent studies (Riediger et al., 2010) the anorectic effect of LPS was paralleled by a decrease in energy expenditure and RQ. These responses were also partly attenuated by central iNOS blockade. For experimental reasons we did not try to reproduce the telemetric measurements of physical activity and body temperature that we conducted in our recent study (Riediger et al., 2010). Although we cannot exclude direct effects of LPS on energy expenditure, the drop in metabolic rate may at least partly be secondary to a decrease in locomotor activity and a decrease in the specific dynamic action of food intake on energy expenditure. The LPS-induced decrease in energy intake caused a drop in RQ reflecting increased lipid oxidation. The attenuation of this response by 1400W is in line with the beneficial effect of iNOS inhibition on the general energy status because it suggests a preservation of internal energy stores. Notably, our data are consistent with previous studies reporting a decreased RQ value after LPS treatment under comparable experimental conditions (Hollis et al., 2011; Hollis et al., 2010). To our knowledge there are no published studies testing the effect of LPS on RQ values in food deprived rats. In principle, such studies

would exclude the possible influence of treatment-induced changes in energy intake on RQ values. However, food restriction or food deprivation per se introduces confounding factors that limit the interpretation of RQ values under such conditions. The RQ of food-deprived rats decreases to low values, which might make it difficult to detect a treatment-induced decline in RQ. Furthermore, food deprivation increases food-seeking behavior at least in healthy animals, which has an impact on metabolism that is difficult to control for in LPS treated rats. Within the framework of our objectives, we did not intend to exclude any determinants contributing to changes in RQ under physiological ad libitum feeding conditions. Therefore, and because of the experimental limitations described above we did not include studies using food-deprived animals.

Overall, a pharmacological iNOS inhibition might be a possible approach to treat disease-related anorexia under chronic conditions either as a single drug treatment or in combination with other anti-anorectic agents. Particularly our current demonstration that inflammatory NO signaling inhibits the target cells for the orexigenic hormone ghrelin might be of crucial importance in this context. Earlier studies pointed to a possible use of ghrelin or ghrelin mimetics as an anti-anorectic agent for the treatment of different forms of sickness anorexia (DeBoer et al., 2007; Hanada et al., 2003; Hataya et al., 2003; Nagaya et al., 2005; Neary et al., 2004; Strassburg et al., 2008). The synthetic ghrelin analog (BIM-28131) exerted positive effects on food intake and body weight in healthy and in tumor-bearing rats (Langhans, 2000; Strassburg et al., 2008). Moreover it improved lean body mass in a rat model of chronic kidney disease, which is associated with increased inflammatory cytokines and cachectic weight loss (Deboer et al., 2008). In human studies the safety, tolerability and pharmacokinetics of ghrelin as a possible anti-anorectic/anti-cachectic drug has been evaluated. Ghrelin treatment is well tolerated and safe in cancer patients, but it did not significantly improve food intake and body weight relative to placebo treatment (Strasser et

al., 2008). However, the negative efficacy data of the human studies using ghrelin treatment have to be interpreted with caution due to variables that might need to be better controlled. Other clinical phase I and phase II trials confirmed a positive effect of an oral ghrelin mimetic (RC-1291) on food intake and body weight in both healthy volunteers and cancer patients (Garcia et al., 2007; Garcia and Polvino, 2007).

Although the general outcome of existing preclinical and clinical studies evaluating the treatment with ghrelin or ghrelin mimetics are promising, cancer anorexia/cachexia seems to be associated with decreased ghrelin responsiveness which might limit the efficacy ghrelin dependant therapeutic approaches. Under most but not all tumor-induced cachectic conditions ghrelin levels are elevated, but this increase in the circulating hormone concentration of active ghrelin is not reflected in an increase in energy intake that may be expected (Garcia et al., 2005). Therefore it has been postulated that tumor anorexia might be associated with a partial decrease in ghrelin responsiveness, which appears to be in line with the reduced effectiveness of centrally administered ghrelin to increase food intake in tumor-bearing rats compared to non-tumor-bearing controls (Wisse et al., 2001). The mechanism underlying this decreased ghrelin responsiveness is unknown. Our previous and novel findings showing a NO-dependent inhibition of ghrelin-sensitive neurons under inflammatory conditions suggest a causal relationship between NO and decreased ghrelin responsiveness. Therefore, a combination treatment with ghrelin receptor agonists and iNOS inhibition is likely to result in improved treatment outcome.

In summary our study further substantiates the concept that neuroinflammatory NO signaling plays a crucial role in the mediation of sickness-related anorexia. Orexigenic Arc neurons are inhibited by endogenous iNOS-dependent NO formation in response to LPS-induced inflammation. Moreover, LPS triggers intracellular pathways in the Arc that are involved in iNOS gene transcription (STAT1) and in NO signaling (cGMP). iNOS inhibition might be

pharmacological approach to treat disease-related anorexia or to improve the treatment success of other therapeutic strategies.

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Figure legends:

Figure 1: Effect of iNOS blockade on the electrical activity of ghrelin-excited Arc neurons from an LPS treated (100 μg/kg) (A) and a saline treated control rat (B). The iNOS inhibitor 1400W increased the firing rate after LPS treatment but not under control conditions. NO responsiveness of the 1400W insensitive neuron was confirmed by the inhibitory effect NO donor SNP. Firing rate was increased by ghrelin.

Figure 2: Mean baseline activity before 1400W administration of 1400W-insensitive and 1400W-excited Arc neurons after in vivo stimulation with LPS. Neurons that increased their firing rate in response to pharmacological iNOS inhibition displayed a significantly lower baseline spontaneous activity than 1400W unresponsive Arc neurons (Mann-Whitney-Wilcoxon Test, $p < 0.05$). The dashed-line represents the mean spontaneous baseline activity of in vivo saline pre-treated neurons.

Figure 3: Responsiveness of Arc neurons to the iNOS inhibitor 1400W after saline or LPS treatment (A) and after in vitro incubation of Arc slices without or with LPS (B). The number of 1400W-excited neurons was significantly increased after in vivo or in vitro incubation with LPS (Fisher Exact Test, * $p < 0.05$, ** $p < 0.01$). A blockade of prostaglandin synthesis by co-incubation with the COX inhibitor indomethacin did not change the excitatory effect of 1400W on Arc neurons after in vitro stimulation with LPS (C).

Figure 4: Effect of iNOS blockade on the electrical activity of ghrelin-excited Arc neurons after 4 h LPS incubation (100 ng/ml) (A) or control incubation without LPS (B). The iNOS inhibitor 1400W increased the firing rate after LPS incubation but not under control conditions. NO responsiveness was confirmed by the NO donor SNP.

Figure 5: Responsiveness of Arc neurons to the iNOS inhibitor 1400W after 4 h LPS incubation (100 ng/ml) in the presence (A) or absence (B) of indomethacin (10^{-5} M). 1400W induces excitatory responses in both cases. NO responsiveness was confirmed by the NO donor SNP.

Figure 6: Effect of iNOS blockade on LPS-induced cGMP formation in the Arc. Representative immunostainings of the Arc showing the effect of 4 h in vitro incubation with LPS (100 ng/ml) on cGMP formation in the absence (A, B) or presence (C, D) of the iNOS inhibitor 1400W (10^{-4} M). Scale bar = 100 μm. (E) Quantitative analysis 4 h in vitro incubation with LPS (100 ng/ml) significantly increased the number of cGMP immunoreactive Arc neurons. This response was blocked by the iNOS inhibitor 1400W (10^{-4} M). Different letters indicate statistical differences between groups (one-way ANOVA $F(3,12) = 12.77$, $p = 0.0005$; Newman-Keuls Multiple Comparison Test $p < 0.01$).

Figure 7: Representative immunostainings of the Arc of mice showing LPS-induced pSTAT1 response. Scale bar = 200 μm. Peripheral LPS treatment (40 μg/mouse) significantly increased the number of pSTAT1 immunoreactive cells in the Arc of mice 4h after injection (unpaired t-test; $p < 0.05$).

Figure 8: Effect of central iNOS inhibition on LPS-induced reduction in dark phase food intake (A+B), energy expenditure (C+D) and RQ (E+F). When compared to control conditions the LPS-dependent reductions in these parameters were significantly attenuated by central infusion of 1400W. (t-test; * $p < 0.05$, ** $p < 0.01$, n = 3-4). See text for explanation concerning absolute RQ values.

References

Abram, M., Vu kovic, D., Wraber, B., Doric, M., 2000. Plasma cytokine response in mice with bacterial infection. Mediators Inflammation 9, 229-234.

Becskei, C., Lutz, T.A., Riediger, T., 2010. Reduced fasting-induced activation of hypothalamic arcuate neurons is associated with hyperleptinemia and increased leptin sensitivity in obese mice. Am. J. Physiol.: Regul., Integr. Comp. Physiol. 299, R632-641.

Becskei, C., Riediger, T., Hernadfalvy, N., Arsenijevic, D., Lutz, T.A., Langhans, W., 2008. Inhibitory effects of lipopolysaccharide on hypothalamic nuclei implicated in the control of food intake. Brain, Behav., Immun. 22, 56-64.

Chen, C.C., Chiu, K.T., Sun, Y.T., Chen, W.C., 1999. Role of the cyclic AMP-protein kinase A pathway in lipopolysaccharide-induced nitric oxide synthase expression in RAW 264.7 macrophages. Involvement of cyclooxygenase-2. J. Biol. Chem. 274, 31559-31564.

Cowley, M.A., Smith, R.G., Diano, S., Tschop, M., Pronchuk, N., Grove, K.L., Strasburger, C.J., Bidlingmaier, M., Esterman, M., Heiman, M.L., Garcia-Segura, L.M., Nillni, E.A., Mendez, P., Low, M.J., Sotonyi, P., Friedman, J.M., Liu, H., Pinto, S., Colmers, W.F., Cone, R.D., Horvath, T.L., 2003. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37, 649-661.

De Luca, B., Monda, M., Sullo, A., 1995. Changes in eating behavior and thermogenic activity following inhibition of nitric oxide formation. Am. J. Physiol. 268, R1533-1538.

Deboer, M.D., Zhu, X., Levasseur, P.R., Inui, A., Hu, Z., Han, G., Mitch, W.E., Taylor, J.E., Halem, H.A., Dong, J.Z., Datta, R., Culler, M.D., Marks, D.L., 2008. Ghrelin treatment of chronic kidney disease: improvements in lean body mass and cytokine profile. Endocrinology 149, 827-835.

DeBoer, M.D., Zhu, X.X., Levasseur, P., Meguid, M.M., Suzuki, S., Inui, A., Taylor, J.E., Halem, H.A., Dong, J.Z., Datta, R., Culler, M.D., Marks, D.L., 2007. Ghrelin treatment causes increased food intake and retention of lean body mass in a rat model of cancer cachexia. Endocrinology 148, 3004-3012.

Dell'Albani, P., Santangelo, R., Torrisi, L., Nicoletti, V.G., de Vellis, J., Giuffrida Stella, A.M., 2001. JAK/STAT signaling pathway mediates cytokine-induced iNOS expression in primary astroglial cell cultures. J. Neurosci. Res. 65, 417-424.

Dickson, S.L., Luckman, S.M., 1997. Induction of c-fos messenger ribonucleic acid in neuropeptide Y and growth hormone (GH)-releasing factor neurons in the rat arcuate nucleus

following systemic injection of the GH secretagogue, GH-releasing peptide-6. Endocrinology 138, 771-777.

Edwards, C.M., Abusnana, S., Sunter, D., Murphy, K.G., Ghatei, M.A., Bloom, S.R., 1999. The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and galanin. J. Endocrinol. 160, R7-12.

Farr, S.A., Banks, W.A., Kumar, V.B., Morley, J.E., 2005. Orexin-A-induced feeding is dependent on nitric oxide. Peptides 26, 759-765.

Feil, R., Kleppisch, T., 2008. NO/cGMP-dependent modulation of synaptic transmission. Handb. Exp. Pharmacol., 529-560.

Garcia, J.M., Boccia, R.V., Graham, C., Kumor, K., Polvino, W.J., 2007. A phase II randomized, placebo-controlled, double-blind study of the efficacy abd safety of RC-1291 (RC) for the treatment of cancer cachexia. J. Clin. Oncol. 25, 9133.

Garcia, J.M., Garcia-Touza, M., Hijazi, R.A., Taffet, G., Epner, D., Mann, D., Smith, R.G., Cunningham, G.R., Marcelli, M., 2005. Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia. J. Clin. Endocrinol. Metab. 90, 2920-2926.

Garcia, J.M., Polvino, W.J., 2007. Effect on body weight and safety of RC-1291, a novel, orally available ghrelin mimetic and growth hormone secretagogue: results of a phase I, randomized, placebo-controlled, multiple-dose study in healthy volunteers. Oncologist 12, 594-600.

Garvey, E.P., Oplinger, J.A., Furfine, E.S., Kiff, R.J., Laszlo, F., Whittle, B.J., Knowles, R.G., 1997. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. J. Biol. Chem. 272, 4959-4963.

Gaskin, F.S., Farr, S.A., Banks, W.A., Kumar, V.B., Morley, J.E., 2003. Ghrelin-induced feeding is dependent on nitric oxide. Peptides 24, 913-918.

Gautron, L., Lafon, P., Chaigniau, M., Tramu, G., Laye, S., 2002. Spatiotemporal analysis of signal transducer and activator of transcription 3 activation in rat brain astrocytes and pituitary following peripheral immune challenge. Neuroscience 112, 717-729.

Guo, Z., Shao, L., Du, Q., Park, K.S., Geller, D.A., 2007. Identification of a classic cytokine-induced enhancer upstream in the human iNOS promoter. Faseb J. 21, 535-542.

Hanada, T., Toshinai, K., Kajimura, N., Nara-Ashizawa, N., Tsukada, T., Hayashi, Y., Osuye, K., Kangawa, K., Matsukura, S., Nakazato, M., 2003. Anti-cachectic effect of ghrelin in nude mice bearing human melanoma cells. Biochem. Biophys. Res. Commun. 301, 275-279.

Hart, B.L., 1988. Biological basis of the behavior of sick animals. Neurosci. Biobehav. Rev. 12, 123-137.

Hataya, Y., Akamizu, T., Hosoda, H., Kanamoto, N., Moriyama, K., Kangawa, K., Takaya, K., Nakao, K., 2003. Alterations of plasma ghrelin levels in rats with lipopolysaccharide-induced wasting syndrome and effects of ghrelin treatment on the syndrome. Endocrinology 144, 5365-5371.

Hollis, J.H., Jonaidi, H., Lemus, M., Oldfield, B.J., 2011. The endocannabinoid arachidonylethanolamide attenuates aspects of lipopolysaccharide-induced changes in energy intake, energy expenditure and hypothalamic Fos expression. J. Neuroimmunol. 233, 127-134.

Hollis, J.H., Lemus, M., Evetts, M.J., Oldfield, B.J., 2010. Central interleukin-10 attenuates lipopolysaccharide-induced changes in food intake, energy expenditure and hypothalamic Fos expression. Neuropharmacology 58, 730-738.

Jansky, L., Vybiral, S., Pospisilova, D., Roth, J., Dornand, J., Zeisberger, E., Kaminkova, J., 1995. Production of systemic and hypothalamic cytokines during the early phase of endotoxin fever. Neuroendocrinology 62, 55-61.

Kim, O.S., Park, E.J., Joe, E.H., Jou, I., 2002. JAK-STAT signaling mediates gangliosides-induced inflammatory responses in brain microglial cells. J. Biol. Chem. 277, 40594-40601.

Kleinert, H., Wallerath, T., Fritz, G., Ihrig-Biedert, I., Rodriguez-Pascual, F., Geller, D.A., Forstermann, U., 1998. Cytokine induction of NO synthase II in human DLD-1 cells: roles of the JAK-STAT, AP-1 and NF-kappaB-signaling pathways. Br. J. Pharmacol. 125, 193-201.

Kobayashi, Y., 2010. The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. J. Leukoc. Biol. 88, 1157-1162.

Kohno, D., Gao, H.Z., Muroya, S., Kikuyama, S., Yada, T., 2003. Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca2+ signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. Diabetes 52, 948-956.

Konsman, J.P., Vigues, S., Mackerlova, L., Bristow, A., Blomqvist, A., 2004. Rat brain vascular distribution of interleukin-1 type-1 receptor immunoreactivity: relationship to patterns of inducible cyclooxygenase expression by peripheral inflammatory stimuli. J. Comp. Neurol. 472, 113-129.

Langhans, W., 2000. Anorexia of infection: current prospects. Nutrition 16, 996-1005.

Langhans, W., 2007. Signals generating anorexia during acute illness. Proc. Nutr. Soc. 66, 321-330.

Langhans, W., Harlacher, R., Scharrer, E., 1989. Verapamil and indomethacin attenuate endotoxin-induced anorexia. Physiol. Behav. 46, 535-539.

Langhans, W., Savoldelli, D., Weingarten, S., 1993. Comparison of the feeding responses to bacterial lipopolysaccharide and interleukin-1 beta. Physiol. Behav 53, 643-649.

Lim, C.P., Cao, X., 2006. Structure, function, and regulation of STAT proteins. Mol. Biosyst. 2, 536-550.

Lugarini, F., Hrupka, B.J., Schwartz, G.J., Plata-Salaman, C.R., Langhans, W., 2002. A role for cyclooxygenase-2 in lipopolysaccharide-induced anorexia in rats. Am. J. Physiol.: Regul., Integr. Comp. Physiol. 283, R862-868.

Morley, J.E., Alshaher, M.M., Farr, S.A., Flood, J.F., Kumar, V.B., 1999. Leptin and neuropeptide Y (NPY) modulate nitric oxide synthase: further evidence for a role of nitric oxide in feeding. Peptides 20, 595-600.

Morley, J.E., Farr, S.A., Sell, R.L., Hileman, S.M., Banks, W.A., 2011. Nitric oxide is a central component in neuropeptide regulation of appetite. Peptides 32, 776-780.

Nagaya, N., Itoh, T., Murakami, S., Oya, H., Uematsu, M., Miyatake, K., Kangawa, K., 2005. Treatment of cachexia with ghrelin in patients with COPD. Chest 128, 1187-1193.

Neary, N.M., Small, C.J., Wren, A.M., Lee, J.L., Druce, M.R., Palmieri, C., Frost, G.S., Ghatei, M.A., Coombes, R.C., Bloom, S.R., 2004. Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. J. Clin. Endocrinol. Metab. 89, 2832-2836.

Paxinos, G., Watson, C., 2007. The rat brain in stereotaxic coordinates. Academic Press/Elsevier, Amsterdam; Boston.

Pecchi, E., Dallaporta, M., Jean, A., Thirion, S., Troadec, J.D., 2009. Prostaglandins and sickness behavior: old story, new insights. Physiol. Behav. 97, 279-292.

Plata-Salaman, C.R., 1996. Anorexia during acute and chronic disease. Nutrition 12, 69-78.

Riediger, T., Bothe, C., Becskei, C., Lutz, T.A., 2004. Peptide YY directly inhibits ghrelin-activated neurons of the arcuate nucleus and reverses fasting-induced c-Fos expression. Neuroendocrinology 79, 317-326.

Riediger, T., Cordani, C., Potes, C.S., Lutz, T.A., 2010. Involvement of nitric oxide in lipopolysaccharide induced anorexia. Pharmacol. Biochem. Behav. 97, 112-120.

Riediger, T., Giannini, P., Erguven, E., Lutz, T., 2006. Nitric oxide directly inhibits ghrelin-activated neurons of the arcuate nucleus. Brain. Res. 1125, 37-45.

Riediger, T., Traebert, M., Schmid, H.A., Scheel, C., Lutz, T.A., Scharrer, E., 2003. Site-specific effects of ghrelin on the neuronal activity in the hypothalamic arcuate nucleus. Neurosci. Lett. 341, 151-155.

Rummel, C., Sachot, C., Poole, S., Luheshi, G.N., 2006. Circulating interleukin-6 induces fever through a STAT3-linked activation of COX-2 in the brain. Am. J. Physiol.: Regul., Integr. Comp. Physiol. 291, R1316-1326.

Rummel, C., Voss, T., Matsumura, K., Korte, S., Gerstberger, R., Roth, J., Hubschle, T., 2005. Nuclear STAT3 translocation in guinea pig and rat brain endothelium during systemic challenge with lipopolysaccharide and interleukin-6. J. Comp. Neurol. 491, 1-14.

Schmidt, H.H., Lohmann, S.M., Walter, U., 1993. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim. Biophys. Acta 1178, 153-175.

Sergeyev, V., Broberger, C., Hokfelt, T., 2001. Effect of LPS administration on the expression of POMC, NPY, galanin, CART and MCH mRNAs in the rat hypothalamus. Brain Res. Mol. Brain. Res. 90, 93-100.

Squadrito, F., Calapai, G., Cucinotta, D., Altavilla, D., Zingarelli, B., Ioculano, M., Urna, G., Sardella, A., Campo, G.M., Caputi, A.P., 1993. Anorectic activity of NG-nitro-L-arginine, an inhibitor of brain nitric oxide synthase, in obese Zucker rats. Eur. J. Pharmacol. 230, 125-128.

Stempelj, M., Kedinger, M., Augenlicht, L., Klampfer, L., 2007. Essential role of the JAK/STAT1 signaling pathway in the expression of inducible nitric-oxide synthase in intestinal epithelial cells and its regulation by butyrate. J. Biol. Chem. 282, 9797-9804.

Strassburg, S., Anker, S.D., Castaneda, T.R., Burget, L., Perez-Tilve, D., Pfluger, P.T., Nogueiras, R., Halem, H., Dong, J.Z., Culler, M.D., Datta, R., Tschop, M.H., 2008. Long-term effects of ghrelin and ghrelin receptor agonists on energy balance in rats. Am. J. Physiol.: Endocrinol. Metab. 295, E78-84.

Strasser, F., Lutz, T.A., Maeder, M.T., Thuerlimann, B., Bueche, D., Tschop, M., Kaufmann, K., Holst, B., Brandle, M., von Moos, R., Demmer, R., Cerny, T., 2008. Safety, tolerability and pharmacokinetics of intravenous ghrelin for cancer-related anorexia/cachexia: a randomised, placebo-controlled, double-blind, double-crossover study. Br. J. Cancer 98, 300-308.

Takeda, K., Akira, S., 2000. STAT family of transcription factors in cytokine-mediated biological responses. Cytokine Growth Factor Rev. 11, 199-207.

Tedeschi, E., Menegazzi, M., Margotto, D., Suzuki, H., Forstermann, U., Kleinert, H., 2003. Anti-inflammatory actions of St. John's wort: inhibition of human inducible nitric-oxide

synthase expression by down-regulating signal transducer and activator of transcription-1alpha (STAT-1alpha) activation. J. Pharmacol. Exp. Ther. 307, 254-261.

Traebert, M., Riediger, T., Whitebread, S., Scharrer, E., Schmid, H.A., 2002. Ghrelin acts on leptin-responsive neurones in the rat arcuate nucleus. J. Neuroendocrinol. 14, 580-586.

Willesen, M.G., Kristensen, P., Romer, J., 1999. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. Neuroendocrinology 70, 306-316.

Wisse, B.E., Frayo, R.S., Schwartz, M.W., Cummings, D.E., 2001. Reversal of cancer anorexia by blockade of central melanocortin receptors in rats. Endocrinology 142, 3292-3301.

Wong, M.L., Rettori, V., al-Shekhlee, A., Bongiorno, P.B., Canteros, G., McCann, S.M., Gold, P.W., Licinio, J., 1996. Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. Nat. Med. 2, 581-584.

Woods, S.C., Seeley, R.J., Porte, D., Jr., Schwartz, M.W., 1998. Signals that regulate food intake and energy homeostasis. Science 280, 1378-1383.

Tables and figures

Table 1

Parameters	In vivo LPS stimulation M ± SEM (n = 6)	In vitro LPS stimulation M ± SEM (n = 4)
Mean spontaneous activity [s]	0.7 ± 0.5	1.6 ± 0.9
Mean latency [s]	428 ± 117	189 ± 68
Absolute response [Hz]	1.5 ± 0.6	1.8 ± 0.5
Absolute peak response [Hz]	2.7 ± 0.9	3.2 ± 0.5
Mean response duration [s]	928 ± 169	1727 ± 64

Table 1: Effect parameters of the excitatory responses induced by 1400W (10^{-4} M, n = 6 and n = 4) in vivo and in vitro respectively.

Figure 1

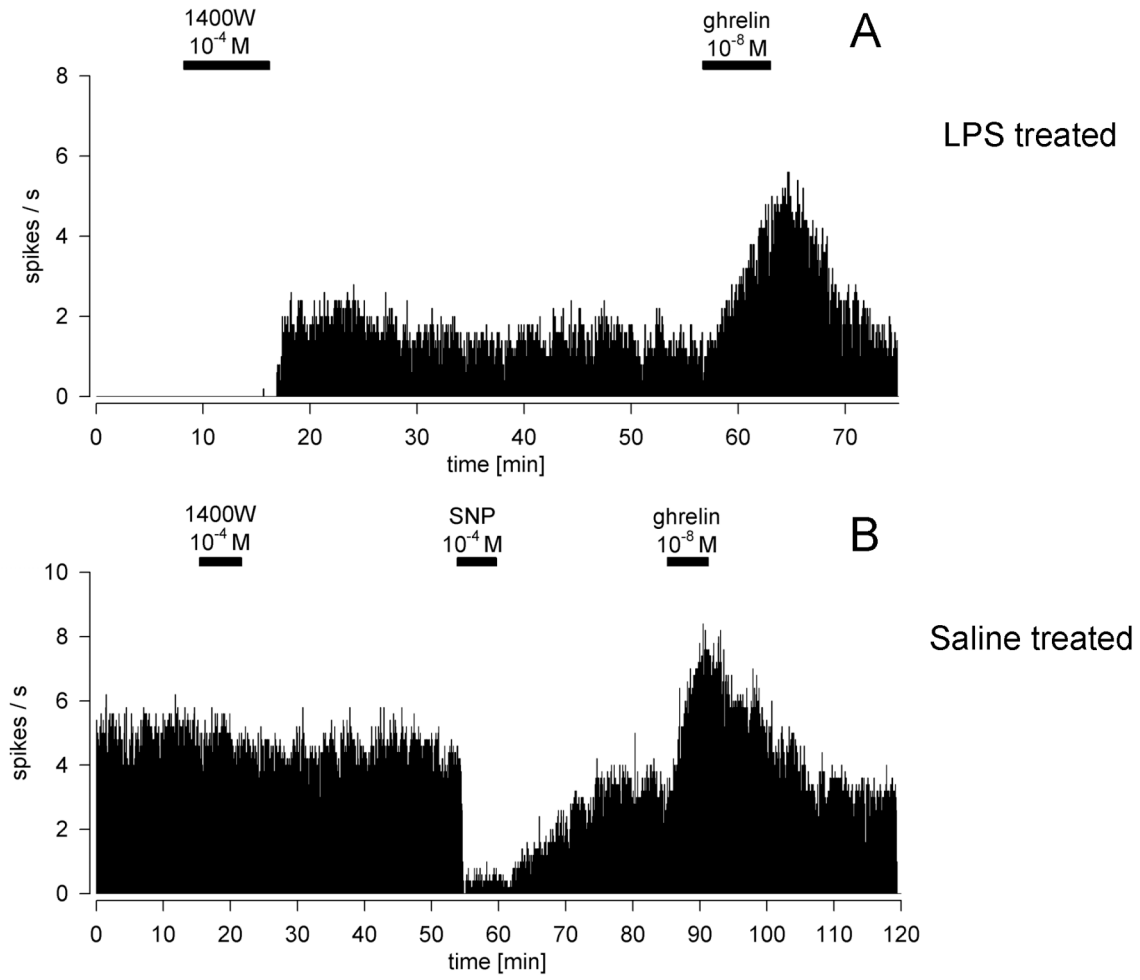

1400W
10-4 M
ghrelin
10-8 M
A
LPS treated
spikes / s
time [min]
1400W
10-4 M
SNP
10-4 M
ghrelin
10-8 M
B
Saline treated
spikes / s
time [min]

Figure 2

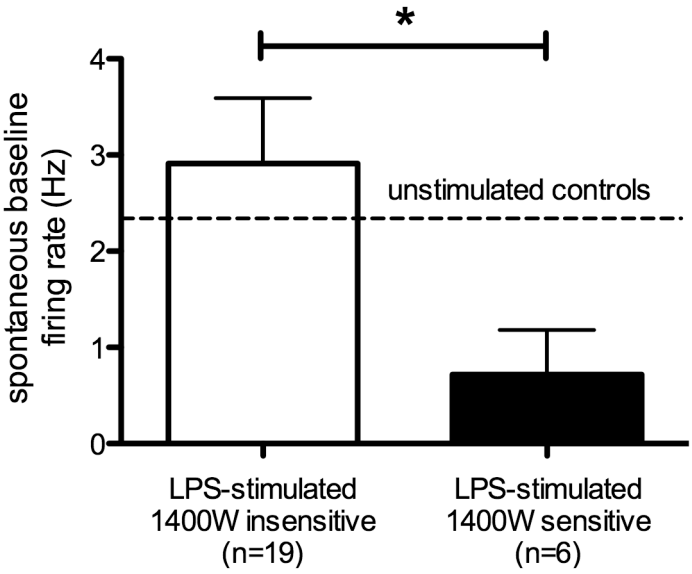

*
4
3
2
1
0
spontaneous baseline
firing rate (Hz)
unstimulated controls
LPS-stimulated
1400W insensitive
(n=19)
LPS-stimulated
1400W sensitive
(n=6)

Figure 3

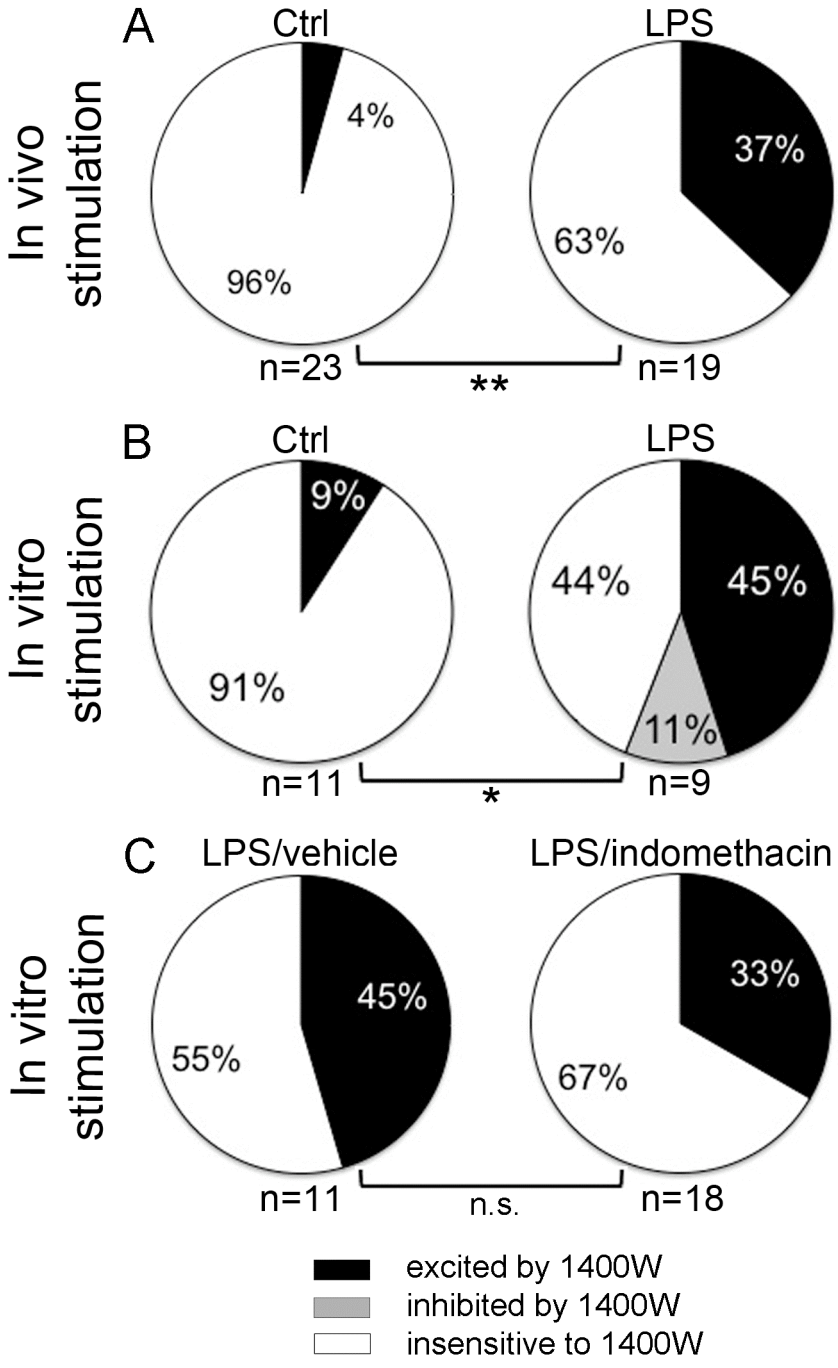

A
Ctrl
LPS
In vivo stimulation
4%
96%
37%
63%
n=23
**
n=19
B
Ctrl
LPS
In vitro stimulation
9%
91%
44%
45%
11%
n=11
*
n=9
C
LPS/vehicle
LPS/indomethacin
In vitro stimulation
45%
55%
33%
67%
n=11
n.s.
n=18
excited by 1400W
inhibited by 1400W
insensitive to 1400W

Figure 4

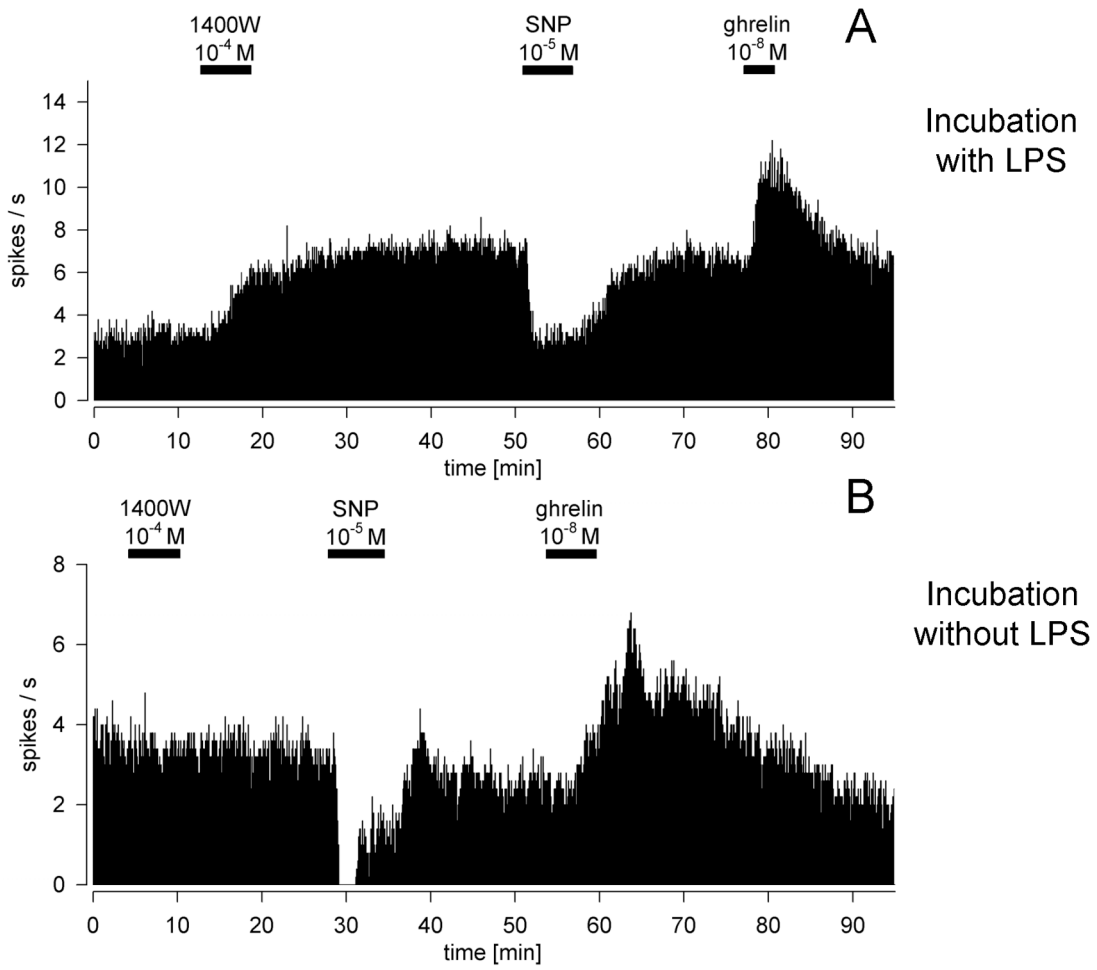

1400W
10-4 M
SNP
10-5 M
ghrelin
10-8 M
A
Incubation
with LPS
spikes / s
time [min]
B
Incubation
without LPS

Figure 5

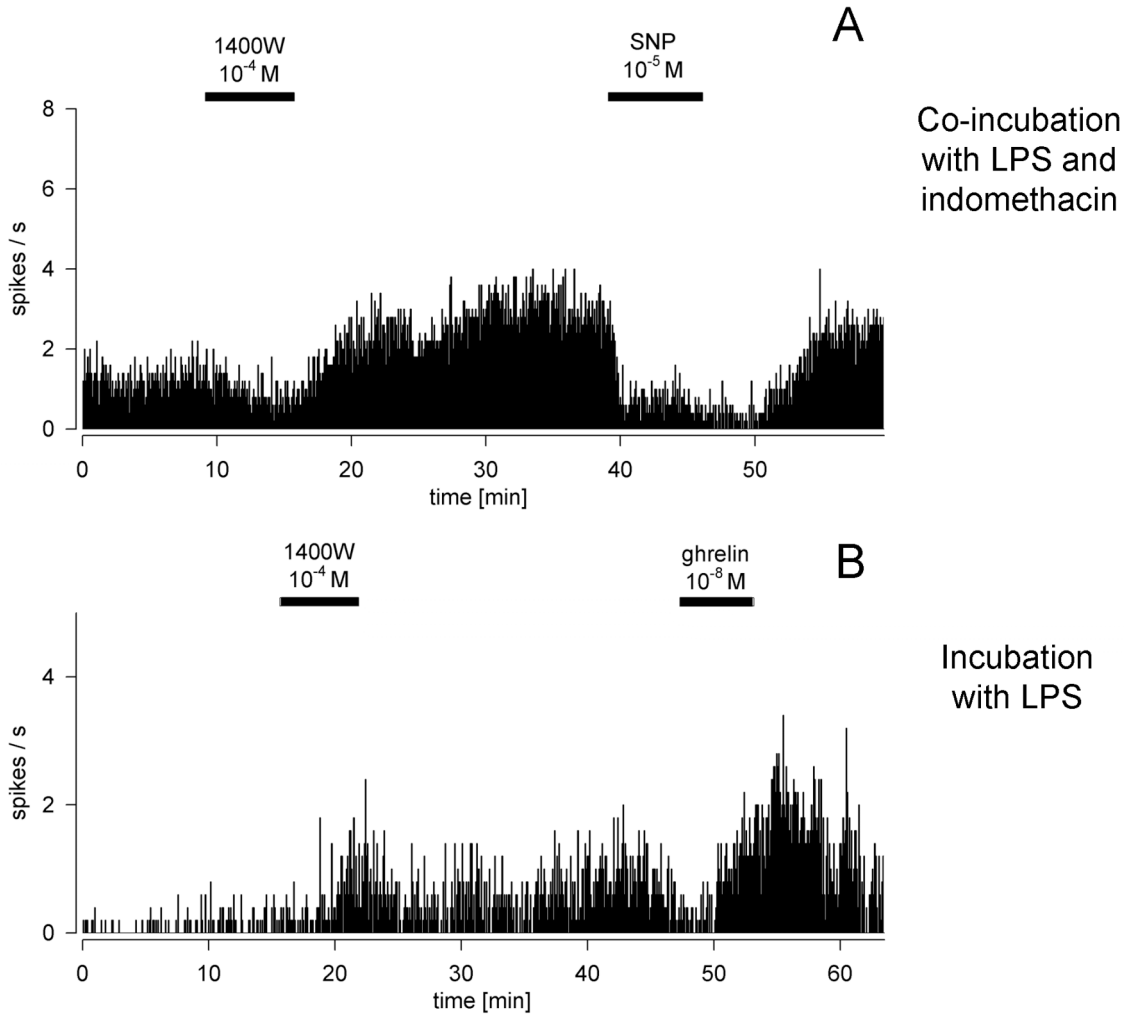
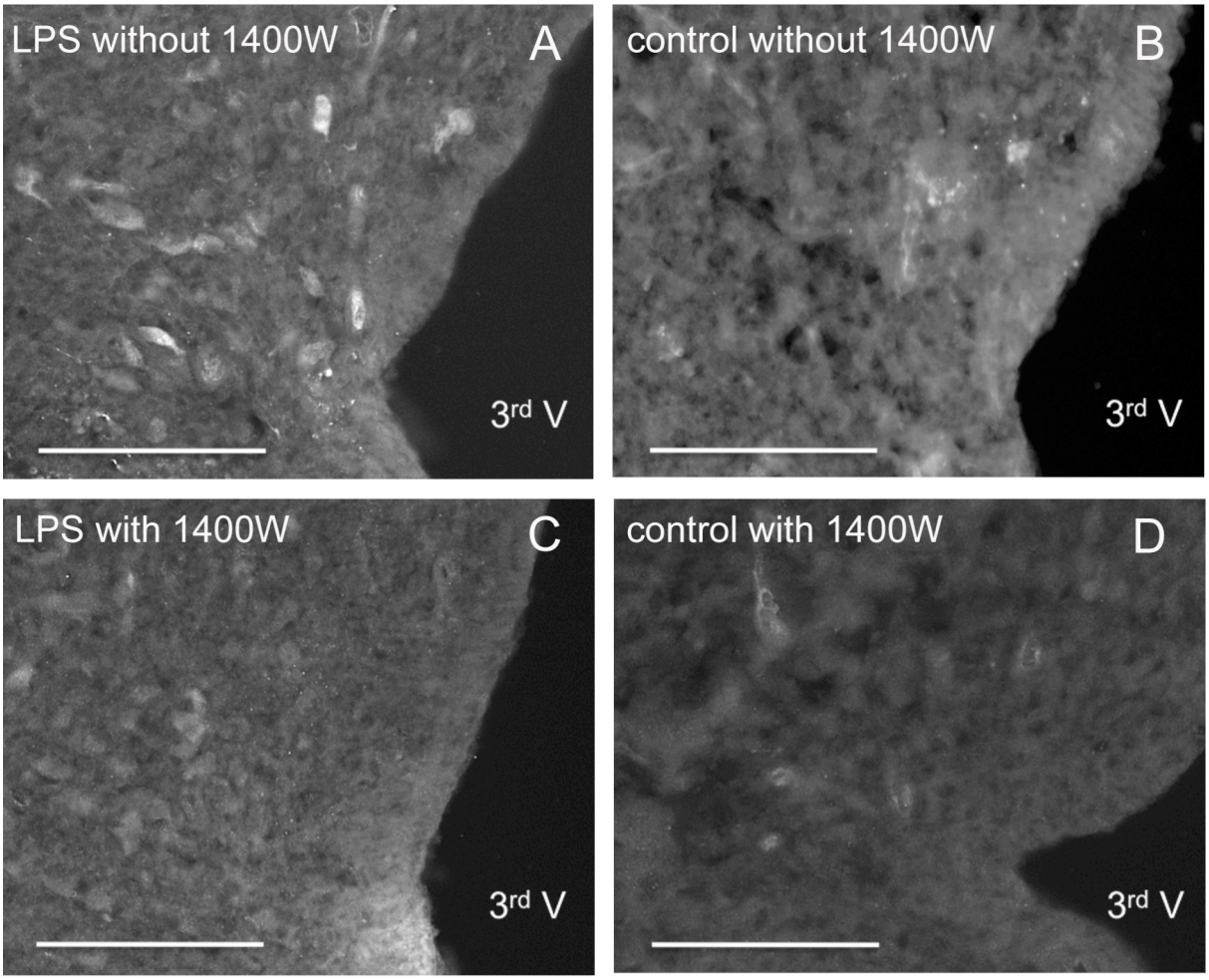

A
1400W
10-4 M
SNP
10-5 M
Co-incubation
with LPS and
indomethacin
spikes / s
time [min]
B
1400W
10-4 M
ghrelin
10-8 M
Incubation
with LPS
spikes / s
time [min]

Figure 6


LPS without 1400W
A
3rd V
control without 1400W
B
3rd V
LPS with 1400W
C
3rd V
control with 1400W
D
3rd V

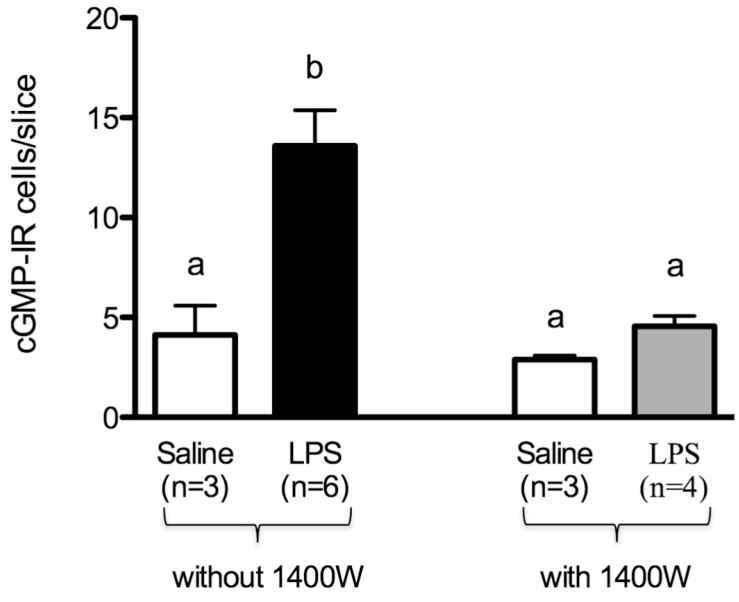

cGMP-IR cells/slice
20
15
10
5
0
a
b
a
a
Saline (n=3)
LPS (n=6)
Saline (n=3)
LPS (n=4)
without 1400W
with 1400W

Figure 7

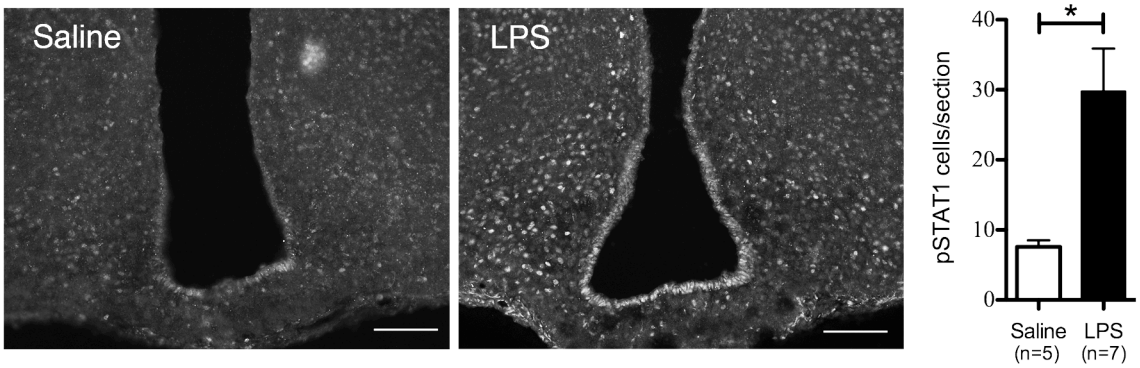
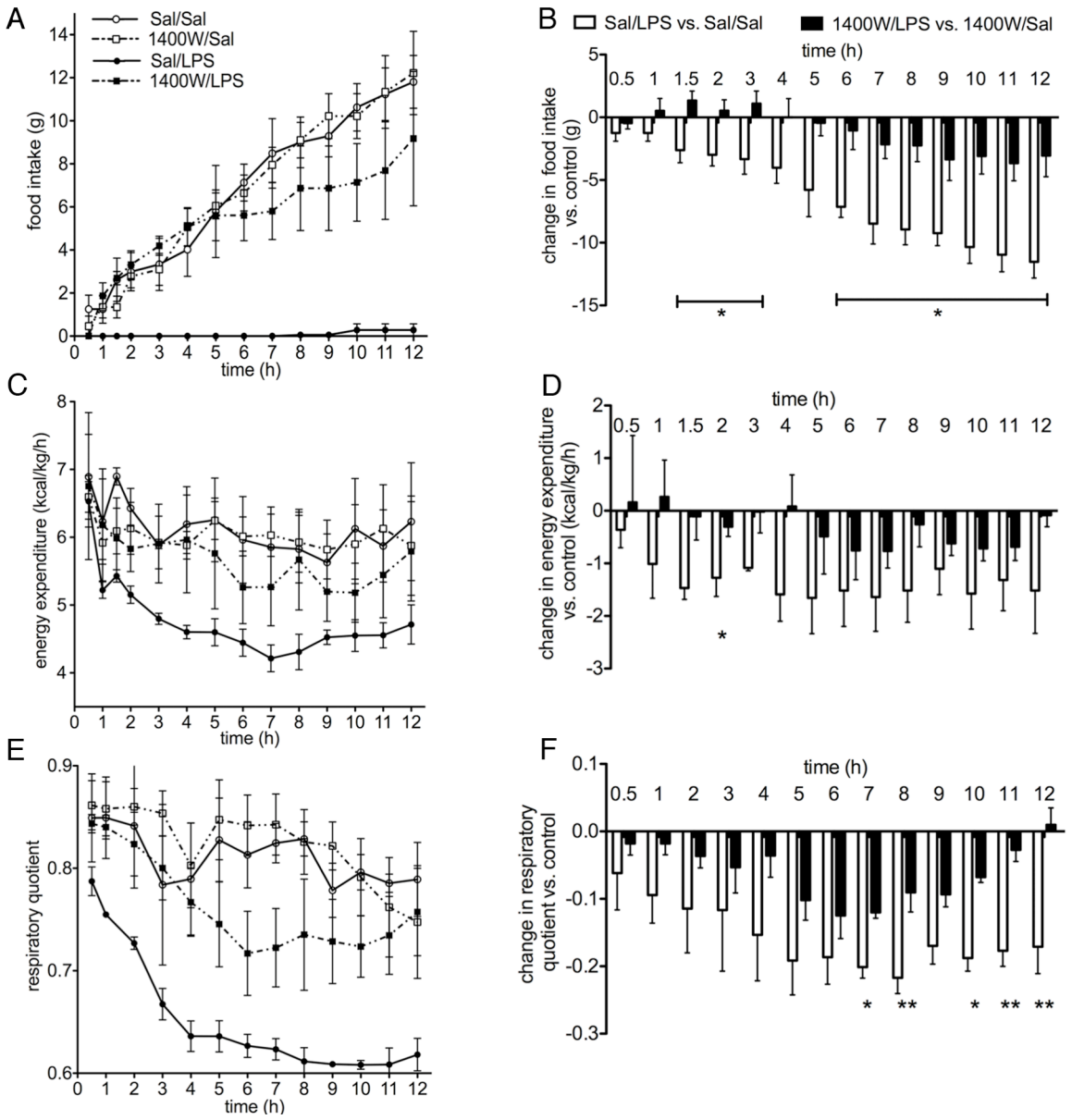


Figure 8


A
Sal/Sal
1400W/Sal
Sal/LPS
1400W/LPS
food intake (g)
time (h)
B
Sal/LPS vs. Sal/Sal
1400W/LPS vs. 1400W/Sal
time (h)
change in food intake vs. control (g)
C
energy expenditure (kcal/kg/h)
time (h)
D
time (h)
change in energy expenditure vs. control (kcal/kg/h)
E
respiratory quotient
time (h)
F
time (h)
change in respiratory quotient vs. control

Curriculum vitae

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08/1989 – 06/2003	Primary school, Gymnasium (High school), Haren (Ems), Germany
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03.07.2009	Graduation from the Veterinary School
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